**18F-FDG Labelled Leukocytes In Vitro Functional Tests: Viability, Chemotaxis and Phagocytosis Assays**

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**Abstract:** *Purpose:* 18F-FDG labelled leukocytes using PET/CT have been suggested as a radiotracer that offer specificity and high quality images for localization of inflammation and infection. The purpose of this *in vitro* study was to determine the functional integrity of leukocytes after labelling with 18F-FDG.

**Materials and Methods:** Leukocytes were separated from whole blood donated by 6 healthy volunteers and labelled with 18F-FDG. The tests included viability, chemotaxis and phagocytosis of the cells.

Trypan Blue was used for viability test. Directional migration was done using chemotaxis under agarose technique. Opsonised zymosan particles were used for phagocytosis assay.

**Results:** Average labelling efficiency (LE) was 90.8%. Viability of 18F-FDG labelled leukocytes one hour after labelling was superior to 99%. Chemotaxis assay showed cells were attracted by chemoattractant after 4 hours of incubation. Labelled cells were found to continue further migration after 24 hours with approximately the same pattern and distance as those of unlabelled cells. Zymosan particles were phagocytosed by the labelled leukocytes.

**Conclusion:** Our study showed that 18F-FDG labelled leukocytes preserved their functional integrity. The labelled cells were viable, responded to inflammatory signals and reacted by engulfing zymosan particles. The results of these *in vitro* tests suggest that 18F-FDG labelled leukocytes could be used for localization of infections and inflammations.

**Keywords:** 18F-FDG, PET/CT, leukocytes, inflammation, infection, chemotaxis, phagocytosis, zymosan.

**INTRODUCTION**

Infection and inflammation are a major concern in clinical practice. Early and accurate detection can help prevent the onset of complications and allows the clinicians to anticipate an effective therapeutic regimen.

Radiolabelling of autologous leukocytes is considered the golden standard for scintigraphic imaging in Nuclear Medicine and remains important for the clinical management of patients with suspected inflammatory disorders.

There are two widely used radiolabelled leukocytes: ¹¹¹In-labelled and ⁹⁹ᵐTc-labelled leukocytes.

Positron emission tomography (PET) is a diagnostic functional imaging technique that uses biologically active molecules as tracer. ¹⁸F-fluorodeoxyglucose (¹⁸F-FDG) has been successfully used for many years to localize abnormal glucose metabolism in oncological, cardiological and neurological patients [1].

FDG, like glucose, is actively transported into the cells by way of glucose transport proteins, particularly Glut-1 and Glut-3. Inside the cells, ¹⁸F-FDG is phosphorylated by hexokinase to 2'-FDG-6-phosphate which cannot enter glycolysis due to the absence of 2'-hydroxyl group. Consequently, FDG-6-phosphate becomes effectively trapped inside the cells and cannot move out [2]. However, by way of the enzyme glucose-6-phosphatase FDG can be partly transported out of the cell again [3]. Once fluorine-18 has undergone radioactive decay to oxygen-18 (stable) the normal metabolic process can proceed.

High uptake of ¹⁸F-FDG has been reported in various inflammation and infection processes [4-11]. The accumulation of deoxyglucose is due to an increased glucose metabolism of activated macrophages and granulocytes in response to inflammatory stimuli [12]. Jones et al. [13] have shown that neutrophils are responsible for the larger part of ¹⁸F-FDG PET signals in both acute and chronic inflammation responses. However, ¹⁸F-FDG is not specific. Its accumulation in malignant tumours and others cells with high glucose uptake limits its use as an inflammation imaging agent.

Utilisation of Positron emission tomography/Computed tomography (PET/CT) has significant advantages over conventional SPECT imaging techniques. It allows precise localization of metabolic abnormalities for more accurate diagnosis. Given the important role of inflammation specific leukocytes and the excellent spatial resolution of positron
Radioactivity in the supernatant and that in the cell suspension were measured in a dose calibrator for the determination of labelling efficiency (LE).

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\text{% labelling efficiency (LE)} = \frac{\text{Radioactivity in cell suspension} \times 100}{\text{Radioactivity in cell suspension} + \text{radioactivity in supernatant}}
\]

**Viability Test**

0.1 mL of labelled cell suspension was transferred into a tube containing 0.25 mL of 0.4% Trypan Blue solution (Sigma-Aldrich, Germany) and 0.15 mL HBSS. The tube was allowed to stand for 5-15 minutes [20] before determining the number of viable cells using haemocytometer. Viable cells do not take up the dye whereas non-viable cells do.

**Chemotaxis Assay Under Agarose [21]**

Cell suspension in HBSS was used for chemotaxis assay. Labelled and unlabelled cells were tested in parallel in order to compare their chemotactic activity.

**Preparation of agarose plate** One percent of agarose (Agarose D-1 LE, Laboratorios Conda, S.A., Madrid, Spain) solution containing 1% of bovine calf serum (Sigma-Aldrich, Germany) was prepared in HBSS and poured onto a 2.5 x 7.5 cm microscope slide. Once solidified, a series of three wells of 3-mm in diameter, 2.5 mm or 5 mm apart was cut using Harris Micro-punch with 3-mm internal diameter (Sigma-Aldrich Logistik GmbH, Germany) and the cored gels were removed by gentle lifting.

**Inoculation of leukocytes, chemoattractant and non-attractant** For each plate, leukocyte suspension was placed in the centre well to nearly full. The two other wells were filled, in the same manner, with the chemoattractant (ZAS) and a non-chemoattractant (PBS). The plates were incubated at 37°C in a humidified atmosphere containing 5% of CO₂ for 4 hours and 24 hours.

**Fixation of leukocytes under agarose** After incubation, the cells were fixed by placing the agarose plate in absolute ethanol followed by 30 minutes in 47% formalin in PBS [21]. Cell fixing in absolute ethanol alone at room temperature overnight was also performed.

**Removal of agarose gel before staining** The agarose gel was gently peeled off from the plate using a small spatula. The cells remained attached onto the plate ready to be stained.

**Staining with Diff-Quik** (Medion Diagnostics AG, Switzerland) Staining was done at room temperature by dipping the plate for 30 seconds each in Fixing Solution, Stain Solution I and Stain Solution II respectively. The plate was drained after each dip. The blue background was then removed by rinsing the plate in distilled water.

**Phagocytosis**

**Preparation of opsonized zymosan** After centrifugation in the section "Preparation of chemoattractant" the zymosan pellet was collected and washed twice with PBS and resuspended in 0.2 mL of PBS.

**Incubation of ¹⁸⁹-FDG labelled leukocytes with opsonized zymosan** Fifty microlitres of labelled leukocyte suspension was mixed with 5 μL of opsonized zymosan preparation and the mixture was incubated at 37°C for 45 minutes. Phagocytosis was examined under a light microscope.

The purpose of this study was to assess the feasibility of leukocytes labelling using ¹⁸⁹-FDG and to test thereafter the integrity of the labelled cells.

**METHODS**

**Labelling of Leukocytes with ¹⁸⁹-FDG**

**Subjects** Six healthy volunteers (3 males, 3 females; age 21-54 years) were asked to fast for at least 6 hours before blood collection which usually took place early in the morning. All blood samples were labelled and subjected to all tests in the same manner.

The labelling method used in our studies was adapted from that described by Dumarey et al. in 2006 [18]. Sixty millilitres of venous blood was collected into a 60 mL syringe containing 600 IU of heparin as an anticoagulant.

Four millilitres of the blood was transferred into a 14 mL tube and centrifuged for 10 minutes at 2000xg to obtain cell-free plasma. The remaining blood was mixed with 12 mL of 6% hydroxyethyl starch (HES) to facilitate the sedimentation of red blood cells. The tube was then allowed to stand at room temperature for 45-90 minutes. The clear plasma was removed by gentle lifting.

The agarose gel was examined under a light microscope.
Stability test 200 μL of cell-free plasma was added to 0.5 mL of labelled leukocytes, kept at 20°C for 4 hours and centrifuged at 2000xg for 10 minutes. Radioactivity in labelled leukocytes and that in the supernatant were measured in a dose calibrator and calculated for radioactivity that was eluted from the labelled cells.

\[
\text{% of Radioactivity eluted} = \frac{\text{Radioactivity in supernatant}}{\text{Radioactivity in labelled leukocytes + radioactivity in supernatant}} \times 100
\]

RESULTS

Labelling efficiency (LE) Average labelling efficiency was 90.8% (89.0% - 92.5%, n = 6).

Viability At 20°C, one hour after labelling, viability of \(^{18}\text{F}-\text{FDG}\) labelled leukocytes in platelet-poor plasma was superior to 99%.

Chemotaxis assay Fig. (1) shows the chemotaxis of \(^{18}\text{F}-\text{FDG}\) labelled leukocytes towards chemoattractant (ZAS) after 4 hours of incubation at 37°C in a humidified atmosphere containing 5% CO\(_2\). There was some random migration towards PBS. Also shown are migration viewed under microscope and a close-up view of leukocytes which composed mainly of polymorphonuclear leukocytes (PMNL). Migration of \(^{18}\text{F}-\text{FDG}\) labelled leukocytes and that of unlabelled leukocytes after 24-hour incubation are shown in Fig. (2). The non-sedimented erythrocytes that remained in the labelled leukocyte suspension did not migrate. They can be seen as a dark spot inside the well (Figs. 1, 2).

Phagocytosis assay After 45 minutes of incubation at 37°C, opsonized zymosan particles were ingested by labelled leukocytes as shown in Fig. (3).

Stability Two to three percent of radioactivity was found in the supernatant separated from the cell suspension that had been kept at 20°C for 4 hours.

DISCUSSION AND CONCLUSION

Positron emission tomography has significant advantages over conventional SPECT because of its high spatial resolution and sensitivity. Besides, the results can be obtained within few hours [11, 23]. \(^{18}\text{F}-\text{FDG}\) labelled leukocyte imaging can be performed within 3 to 4 hours after injection compared to 24 hours for \(^{11}\text{In}\) labelled leukocytes.
Labelling efficiency of $^{18}$F-FDG labelled leukocytes can vary considerably from 24% to 96% [18]. This variation is understandable because LE is affected by many factors such as skill or expertise, blood sample, anticoagulant, number of cells, reaction medium, reaction volume, incubation time, temperature, and so on. It also depends on the extent of cell damage that could occur during the labelling process.

Osmon and Danpure [1] in 1992, demonstrated that the extent of $^{18}$F-FDG uptake by granulocytes was inversely proportional to the concentration of glucose in the labelling medium. The number of cells to be labelled is another important factor. McAfee [24], in 1983, stated that “With any indiscriminate agent, the labelling efficiency increases with a greater number of cells in the smallest possible volume”. In our studies, about all volunteers were asked to fast but one had had his breakfast before blood withdrawal. The LE (89.6%) was found to be in line with the others (90.8%). For this study, we had decided to use the techniques that work well with Tc-99m in our laboratory. Cells should be handled very gently throughout the labelling process. Leukocytes must be collected exhaustively from the plasma at the end of the sedimentation period. We have noticed that the result is poor when the plasma volume is small. Better contact between cells and FDG can be increased by shaking and reducing the distance between them. This means that the final reaction volume should be kept very small.

Cell viability, should be examined within 15 minutes as viable cells may take up dye if exposed for extended periods of time [20]. The trypan blue exclusion test is based on the principle that live cells possess intact cell membranes that exclude certain dyes such as trypan blue, whereas dead cells do not [25]. In 2004, Kwok et al. [26] found that trypan blue was toxic to cells and could cause cell death. For this reason, cells should not be left in the dye for longer periods of time.

$^{18}$F-FDG seems to attach firmly to leukocytes as very small radioactivity was eluted into the supernatant. This result can be explained by glycolysis of cells including blood cells [27, 28]. FDG, like glucose, is actively transported into the cell and is phosphorylated by hexokinase. But unlike glucose, the oxygen in C-2 position which is necessary for further glycolysis is replaced by florine. Consequently, FDG-6-phosphate can neither undergo further metabolism nor diffuse out of the cell. It is trapped inside the cell while dephosphorylation occurs slowly [2].

The stability test was carried out at 20°C to determine the rate of elution of $^{18}$F-FDG at room temperature because after dispensing a prompt administration could not be guaranteed. The firm attachment of FDG to leukocytes helps assure that the localization after injection, if any, is that of the labelled cells and not the eluted FDG.

The patterns of migration of the labelled and the unlabelled leukocytes are almost identical regardless of the method of fixation. The only difference between absolute ethanol and absolute ethanol/formalin fixation is the intensity of the stain. We believe that the use of formalin is not indispensable. Ethanol fixation has been chosen for the close-up view in Fig. (1) because the stain is lighter.

Opsonized zymosan particles were found to have been engulfed by labelled neutrophils. This finding demonstrated that $^{18}$F-FDG labelled leukocytes preserved their phagocytic activity which is essential for searching and localizing infections.

$^{18}$F-FDG labelled leukocytes have neither been extensively studied nor officially accepted for routine uses in Nuclear Medicine despite their promised qualities and characteristics. Our results confirmed that after labelling, leukocytes preserved their functional integrity. We do not think that, from a clinical point of view, the short half-time of 18F-FDG is a limiting factor in detecting infection. Indeed, the exam could be performed in two steps: one whole body acquisition classically performed about 60 minutes after the injection, and a delayed specific acquisition, centred on the region of interest, performed 3 to 4 hours after the injection. Technical (new fast and sensitive “time-of-flight” PET) and clinical (diagnostic differentiation of infection/inflammation is possible with a two steps protocol) aspects strongly support the feasibility of 18F-FDG labelled leukocytes in clinical practice [29, 30].

Further studies in patients presenting quality images, specificity and accuracy will help this radiotracer find its place it deserves in Nuclear Medicine.

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